THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. IV. POST-ECDYSIAL HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE HEPATO-PANCREAS AND INTEGUMENTAL TISSUES [1]

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Following molt, the major tasks which confront the crustacean are growth of the soft tissues and the continued accretionary growth and hardening of the skeleton by deposition of mineral salts therein. In spiny lobsters of 80–89 mm. carapace length, weight stability is not achieved until 28–35 days following molt (late Stage C) (Travis, 1954). This is a period at which the skeleton is fully hardened, water content is normal, and presumably growth of the tissues is fairly stable. During the early postmolt period, however, when rapid accretionary growth and calcification of the skeleton are occurring, marked changes are observed in the hepatopancreas and integumental tissues. Accordingly, the present paper will be concerned with those marked changes in the hepatopancreas and integumental tissues which occur concomitantly with the development and calcification of the post-exuvial layers of the skeleton.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

Stages of the molting cycle were designated by time intervals, in days, as previously described (Travis, 1955a) and by the method of Drach (1939). For *Panulirus argus,* Stage A through C encompasses a period of approximately 51 days during the summer months. Stage A, immediately following molt and the stage in which the principal layer begins to be deposited, has a duration of approximately 24 hours or one day. Stage B, beginning calcification, continued thickening of the principal layer and preliminary hardening of the skeleton, is approximately six days

[1] This work was supported in part by an E. L. Mark Fellowship from Harvard University, a Mees Fellowship from the Bermuda Biological Station, and an Atomic Energy Commission Pre- and Postdoctoral Fellowship. The author wishes to express her sincere appreciation to Dr. John H. Welsh, Dr. E. E. Mortensen, and Dr. A. B. Dawson for their most helpful criticism and suggestions regarding this manuscript.

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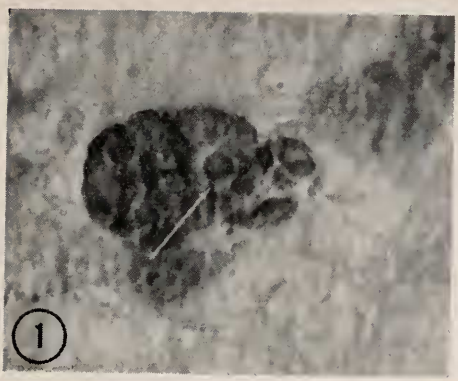
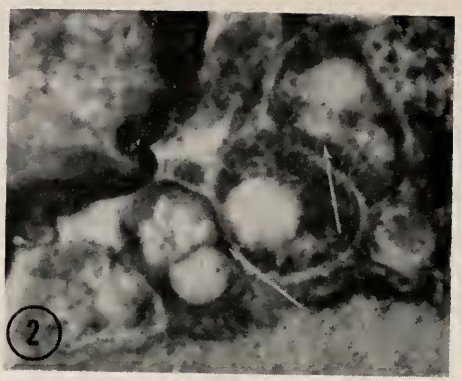
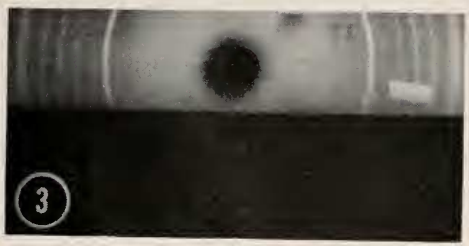
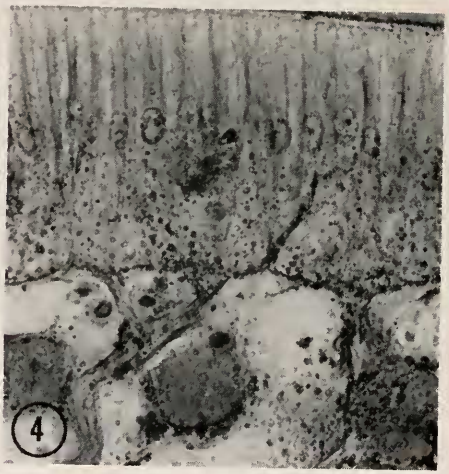
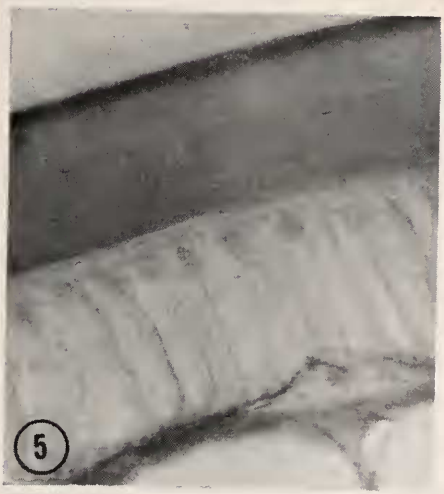
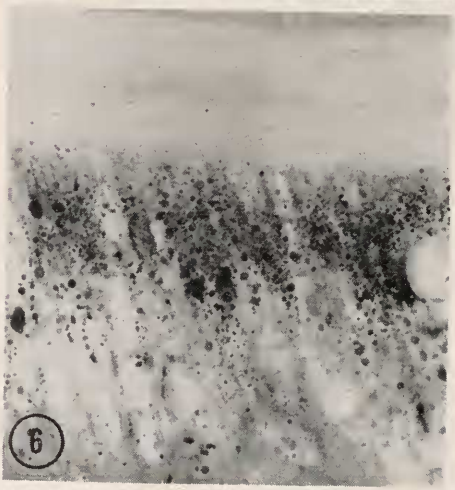












FIGS. 1–6.

in length, existing from two through seven days following molt. Stage C, a stage in which the principal layer and the new membranous layer are completed and in which the skeleton is completely hardened, is the longest period of the molting cycle (44 days), existing from approximately the eighth day through the fifty-first day following molt.

Histological and histochemical methods

For the histological and histochemical studies, pieces of integument and integumental tissues were removed from the carapace of *Panulirus* (see Figure 1; Travis, 1955a) on each of eight consecutive days following molt. Likewise the right posterior lobe of the hepatopancreas was removed on each of seven consecutive days following molt. Tissues from three animals were used to represent each of these days with the exception of the first, fourth, and eighth day following molt for the integumental tissues. In these cases, tissues from one animal were used.

Most integumental tissues were embedded in celloidin and cut at 10 μ. The posterior lobe of the hepatopancreas was embedded in paraffin and cut at 8 μ, with the exception of hepatopancreatic tissues fixed and embedded for lipid detection.

Portions of the integument and hepatopancreas fixed in Helly's and alcoholic Bouin's fluid were stained by the following methods:

1. Mallory's triple stain
2. Periodic acid Schiff (PAS) of McManus, as described by Lillie (1948)
3. Bensley and Bensley's method (1938), for demonstrating muco- or glycoprotein by means of toluidine blue (see Travis, 1955a).

For detection of calcium deposits, portions of the skeleton were fixed in nine parts of 95% alcohol and one part of 40% formaldehyde, and were stained with the following:

1. Mallory's triple stain
2. Schmorl's purpurin (Lillie, 1948)

Figure 1. A reserve cell, "mulberry"-like in appearance, of the sub-epidermal connective tissue from an animal three days following molt. Note lumpy or stainable balls of material (arrow) of mucopolysaccharide as well as calcium. 800 ×.

Figure 2. Large oval reserve cells of the hepatopancreas showing the presence of large vacuoles (arrows), some of which contain flaky or granular-like stainable material while others appear clear, a condition observed from five through seven days following molt and possibly correlated with a marked decrease in mucopolysaccharide and calcium. 800 ×.

Figure 3. X-ray diffraction photograph taken of dry powder obtained from triturated pieces of the area of softening. The presence of calcite lines reveals that calcium carbonate is present in the spiny lobster skeleton as calcite, not aragonite or amorphous calcium carbonate.

Figure 4. Glycogen distribution in the epidermis of the outer integument and sub-epidermal connective tissue at one day postmolt. Note small number of glycogen granules localized in the proximal half of the outer epidermal cells. At this same time abundant amounts are concentrated at the bases of the epidermal cells of the inner integument. 760 ×.

Figure 5. Note that at two days postmolt glycogen has completely disappeared from the epidermis of the inner integument and is not observed again in this tissue during the entire postmolt period. 760 ×.

Figure 6. The heavy concentration of glycogen observed in the distal half of the outer epidermal cells during a period of two through four days following molt. Little glycogen at this time is present in the sub-epidermal connective tissue. 760 ×.

3. Alizarin red S (Manigault, 1939)
4. Von Kossa's method (Lillie, 1948). Before following this procedure, tissues were washed in 5% aqueous KNO_3 for five minutes or more to remove the chloride present. With Von Kossa's method, the silver from the silver nitrate is precipitated as phosphate on the surface of calcium phosphate granules. The silver phosphate is reduced in the presence of light to metallic silver, forming a black crust on the surface of calcium phosphate granules. Mallory (1942) states that calcium carbonate granules become coated with silver carbonate, which in sunlight gives off CO_2 and leaves a black silver oxide on the surface of these granules. These reactions may not occur in the presence of organic substances (Lison, 1953) nor in the presence of quantities of chloride (Lee, 1946).

   Parts of the epicuticle, pigmented layer and principal layer appear either black or brown with Von Kossa's method. Cameron (1930) pointed out that the most recent calcium deposits in teeth appear dark brown by this method, while the older calcified layers appear lighter brown. Some calcified areas, he noted, did not stain at all. It should be pointed out that this method has the great advantage over alizarin and purpurin in that it enables one to visualize calcium deposits in granular form. It is, therefore, excellent for the detection of skeletal deposits whereas the latter methods are not.
5. Microincineration (Scott, 1933) was used to confirm the presence of calcium deposits detected by the stains mentioned above. The white calcium ash under dark-field illimination appeared in the same areas as indicated by the stains. To confirm this as being calcium ash, the gypsum test was used.

   For detection of calcium in the hepatopancreas, tissues were fixed in the same fashion as the integumental tissues and were stained with alizarin red S.

For detection of alkaline phosphatase, integumental and hepatopancreatic tissues were fixed in cold 80% alcohol, and embedded in paraffin. Alkaline phosphatase was determined by the method of Gomori (1941). Control sections were made using the incubating medium without added substrate. This method is extremely useful for the detection of calcium deposits. In control sections which do not show the presence of alkaline phosphatase, calcium deposits, if present, show up remarkably well.

Only the hepatopancreas was used for the detection of lipids. Portions of the tissue, in this case, were fixed in 10% neutral formalin and were imbedded in carbowax (method of Blank and McCarthy, 1950), cut at 10 and 15 μ and stained for lipids with Sudan black B.

Observations

The Postmolt Animal (Stage A and B)

1. *The integument and integumental tissues*

   a. *Tissues*

During the early post-ecdysial period (Stage A and B) pieces of exoskeleton with attached integumental tissues were removed from the lateral portion of the carapace (see Fig. 1; Travis, 1955a). The lateral portions of the carapace of

Crustacea, as one will recall, are folded in such a way that there is an outer epidermis and integument, (the outer integument being in contact with the surrounding sea water) as well as an inner epidermis and integument (the inner integument facing the gills, in contact with the sea water in the branchial chamber). Sections of the exoskeleton with attached integumental tissues indicate that the epidermal cells of the outer integument remain extremely long and attenuated and indeed fibrillar in nature. This condition is similar to that observed in the late premolt animal. The markedly fibrillar nature of the outer epidermis, however, is apparent in all stages of the molting cycle. Nuclei in these epidermal cells of the outer integument are central (Fig. 4) whereas those of the inner integument are more distal in location (Fig. 5). The inner epidermal cells, also somewhat fibrillar in nature, remain about half the length of the outer epidermal cells during all stages of the molting cycle (see figures from Travis, 1955a).

Both the outer and inner epidermis, during the early postmolt period (Stage A and B), show a gamma metachromasia (pink-purple) with toluidine blue, indicating the presence of a glyco- or mucoprotein. The presence of phosphatase, glycogen, and calcium in these tissues during the early post-molt period will be discussed in a subsequent section of this paper.

As was pointed out (Travis, 1955a), the sub-epidermal connective tissue is of a loose spongy type.

The large oval reserve cells, described as "protein cells" by Cuénot (1893) and resembling Leydig Cells, Type I (Kükenthal, 1926–1927), constitute by far the most prominent and most interesting cell types within this sub-epidermal connective tissue. These reserve cells vary greatly in structural appearance during the molting cycle. When storing reserves they become greatly swollen and may take on a "mulberry" appearance (Fig. 1). When devoid of reserves they may decrease in size with their vacuoles becoming clear or containing flaky or granular-like stainable material. Since the reserve cells are found within the tissue spaces among other Leydig cells, they should not, perhaps, be considered as permanent structures within this tissue. This has become somewhat clearer from the work of Sewell (1955), in which he points out that the origin of these reserve cells, which he calls "lipo-protein cells," in *Carcinus* is from amoebocytes and that possibly they revert to amoebocytes following molt. This suggestion could account for their cyclic peaks and declines in size and abundance, and changes in structural appearance at daily intervals during the early postmolt period of *Panulirus,* as indicated below.

For the sake of comparison, the reserve cells in *intermolt animals (late Stage C)* constitute the most prominent cell-types within the connective tissue. They are large oval cells, vesicular in nature, with a capsule-like envelope of cytoplasm and a peripheral nucleus (see figures; Travis, 1955a). They range in size from 24–51 μ with an average size of 32 μ. After alcoholic Bouin's fixation they stain blue-gray with Mallory's triple stain, the vacuoles in this case containing blue-gray flaky or granular-like material; with the PAS method the entire cell is a deep pink-purple color; with toluidine blue these cells stain either blue-gray or green-gray. After A–F (alcoholic formaldehyde) fixation they similarly stain blue-gray with Mallory's triple stain, but do not stain with alizarin red S, purpurin or the Von Kossa method. With these three latter stains the reserve cells could be easily overlooked.

Similarly, for the sake of comparison reserve cells in *premolt animals* (*Stage D*) range in size from 30–51 μ with an average size of around 40 μ. Structurally, they maintain their oval appearance but stainable material within the cells is lumpy and might well be described as consisting of rather discrete spheres (Fig. 1). After alcoholic Bouin's fixation, the cells again stain blue-gray with Mallory's but do not show clearly the speres of stainable material; with PAS they again stain deep pink-purple and show clearly the discrete balls of material; with toluidine blue the balls of material are yellow-green in color and refractile in nature. Following A–F fixation, the cells again fail to stain with alizarin red S, purpurin and the Von Kossa method.

From one through seven days following molt (Stage A and B), the reserve cells appear to undergo cyclic peaks and declines in size and abundance, and the storing of reserves.

At *one day* following molt (Stage A) the reserve cells remain approximately the same size as those observed in the premolt animal, a range in size from 32–48 μ and an average size of 36 μ. However, there would appear to be a slight decrease in number. Following alcoholic Bouin's fixation, they stain in much the same fashion as that observed in the premolt animal, although the spheres of stainable material are not as apparent. They fail to stain, following A–F fixation, with the same stains mentioned in the premolt animal.

On the *second day* following molt (beginning of Stage B) the reserve cells have greatly decreased in number and size. They range in size from 12–29 μ with an average of around 19 μ. When observed, their vacuoles are clear and vesicular, lacking the lumpy balls of material, with the exception of very small spheres at their periphery. They stain in a similar manner to those observed on the first day with the exception of the fact that a few show a very small number of calcium granules after the Von Kossa method.

On the *third day* the reserve cells again are present in great numbers, comparable to the condition observed in the premolt animals. They range in size from 32–45 μ with an average size of around 38 μ, which compares favorably with the average size observed in the pre- or intermolt animal. The reserve cells at this time take on an irregular "mulberry" appearance by enclosing large stainable spheres of material within their vacuoles (Fig. 1). Further, their staining properties change markedly. Following Bouin's fixation, they again stain blue-gray with Mallory's, deep pink-purple with PAS and yellow-green with toluidine blue. Following A–F fixation, they stain for the first time, a brilliant orange-red with Mallory's scarlet with alizarin red S and purpurin, and yellow-brown with Von Kossa's method. This indicates that not only is muco-polysaccharide, possibly muco- or glycoprotein, bound by these cells at this time but that they are filled with calcium, which is distinctly apparent with stains used for this purpose.

Interestingly enough, by *the fourth day* the reserve cells are hardly apparent. The large stainable balls of material are lacking and the few cells present are smaller in size, ranging from 19–29 μ with an average size of about 26 μ. In the few apparent cells, there is little evidence of either mucopolysaccharide material or calcium.

On the *fifth day*, these cells again reach a peak in abundance and size. With all stains and fixatives used, they are similar in every way to the three-day condition, with the exception of the fact that the mucopolysaccharide present stains only faintly with PAS, possibly indicating a decrease in concentration of the muco-

polysaccharide material or the unavailability of its reactive groups to PAS. The cells range in size from 29–42 μ with an average of around 34 μ, are "mulberry-like" in appearance, and are again filled with calcium which shows up after appropriate fixation and staining.

By *six days*, the cells are fewer in number, somewhat smaller (range 17–27 μ, average 23 μ), but similar in staining properties to the fifth-day condition.

On the *seventh day* (end of Stage B) the cells are few in number but are somewhat larger in size (35–40 μ, average about 37 μ), and are detected after Bouin's fixation and toluidine blue staining and following A–F fixation and Mallory's, alizarin red S, purpurin, and Von Kossa's method, indicating again that these cells are loaded with calcium.

By *eight days* following molt (beginning of Stage C), the reserve cells are again not apparent.

It is, therefore, evident that the reserve cells even within the early postmolt period, Stage A and B, undergo, at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties. The polysaccharide material which is distinctly evident up to the fifth day following molt is always diastase-fast and colors deep pink-purple with PAS but does not show gamma metachromasia with toluidine blue. Pearse (1953) has suggested that polysaccharide material staining in this way with PAS and frequently failing to show gamma metachromasia with toluidine blue probably indicates the presence of either a muco- or glycoprotein. As has been mentioned previously (Travis, 1955a) the reserve cells of late Stage C and Stage D animals stain with PAS in the same manner. This polysaccharide material probably represents reserve substances for the new skeleton and may, as was pointed out by Travis (1955a), during the premolt period represent breakdown products from the old skeleton.

None of the integumental tissues of *Panulirus*, unfortunately, were fixed for the detection of lipids. As was pointed out by Travis (1955a) the reserve cells within the connective tissue of the hepatopancreas contain much lipid and it would likewise be expected that the reserve cells of the sub-epidermal tissue also store it. Sewell (1955) has definitely shown that these reserve cells beneath the connective tissue of *Carcinus* do indeed store lipoprotein. These lipoprotein reserves reach a maximum in C_4 (late Stage C) and early D (D_1) and then begin to decrease, apparently as lipid content of the epidermis increases. These cells, as Sewell suggests, begin to disappear after the pre-exuvial layers of the skeleton are deposited and completely disappear by the end of Stage B. However, reserve cells of *Panulirus* become filled with calcium on the third, fifth, sixth, and seventh day following molt whereas on the first, second, and eighth day either no calcium or very little was apparent in the reserve cells. This would suggest to the present author cyclic peaks in calcium storage alternating with cyclic release to the epidermis as calcification of the skeleton occurs. The present author would expect this cyclic process (peaks and declines in size and abundance, changes in structural appearance, and calcium binding and release) to continue throughout early and middle Stage C, since calcification of the skeleton is not fully completed for at least three weeks following molt. If, therefore, the reserve cells arise from amoebocytes, as Sewell (1955) suggests, and possibly revert to amoebocytes after they have discharged their reserves, such a situation could clearly account for the cyclic peaks in size and abundance at varying daily intervals within a single stage of the molting cycle.

In this sense, the present author would be inclined to accept Sewell's suggestion that the reserve cells represent phases of activity of the amoebocyts with peaks not only before molting, as Sewell suggests, but following molt as well. The reserve cell cycle would then correspond more closely with the oenocyte cycle, being present throughout the molting cycle but reaching peaks at various phases of it. It might be pointed out that days in which reserve cells are scarcely apparent (2, 4, and 8 days following molt), large numbers of amoebocytes are apparent in the sub-epidermal connective tissue.

b. *The integument*

During the early postmolt period (Stage A and B) the post-exuvial layers of the skeleton are deposited. Of the post-exuvial layers of the outer integument, only the principal layer or calcified zone is progressively thickened during Stages A and B.

The amount of skeletal material, in total thickness, deposited per day, in the area of carapace from which sections of the integument were cut, during Stages A and B, varies from 14–72 μ with an average of around 38 μ. The thickness of the principal layer when fully formed varies from around 460–550 μ in animals used in this investigation (80–89 mm. carapace length). If one assumes that a constant amount of skeletal material is deposited daily in the principal layer, for example 38 μ, one can, by using this figure and the total thickness of the fully formed principal layer, roughly calculate the time in days when this layer is completed. By using these values, the calculated time at which the principal layer is completed would be around 15 days following molt. This time, however, is in actuality closer to 20 days following molt. Therefore, the membranous or non-calcified layer would not apparently begin to be deposited before the third week following molt (Stage C_3 of Drach, 1939). By the fourth week following molt (late Stage C or C_4 of Drach) the membranous layer is fully formed (Travis, 1955a).

The pigmented layer (toluidine blue-staining) shows the presence of a muco- or glycoprotein one through five days following molt but by the end of this period the tinctorial properties of this layer have decidedly changed. A deep purple rather than a pink-purple is given with this stain, indicating that the properties of the protein and closely associated chitin units of the pigmented zone have been changed by quinones. The newly formed principal layers initially show a green coloration with toluidine blue but shortly take on a pink coloration. The membranous layer, when fully formed, is light green in color with toluidine blue, possibly indicating differences in the organic nature or composition of this and the principal layers.

The inner integument, bordering the gill chamber, undergoes little if any thickening after the second day following molt. It attains a total thickness of approximately 30 μ, that of a late Stage C animal, by the second day following molt, indicating that the inner integument is completed during a period of three days preceding molt and two days following molt (Travis, 1955a).

As the post-exuvial layers are deposited during the early postmolt period (Stage A and B), concomitant hardening of these layers occurs by the deposition of mineral salts therein. It is evident from the analyses of small pieces of the area of softening (Travis, 1955b) that the most abundant mineral constituent in the exoskeleton

is calcium. In order to determine what salts were deposited in the skeleton, further chemical analyses were carried out on the entire area of softening from late Stage C animals. The results of these analyses are indicated in Table I. From these results it is apparent that most of the calcium present in the skeleton is in the form of calcium carbonate. In order to determine whether this is deposited in the organic matrix of the skeleton as amorphous calcium carbonate or as crystalline aragonite or calcite, x-ray diffraction photographs (kindly made by Dr. C. Frondel, Department of Minerology, Harvard University), were taken of dry powder obtained from triturated pieces of the area of softening. These photographs (Fig. 3) indicate that calcium carbonate exists in the spiny lobster skeleton as calcite.

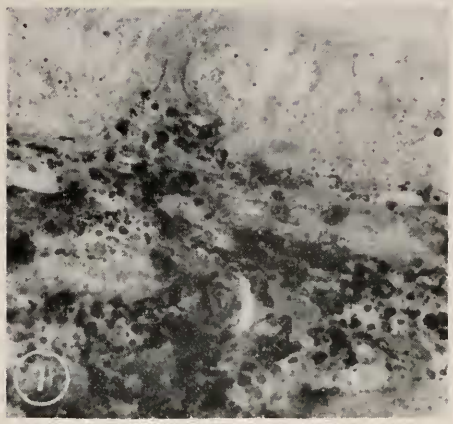
Since the basic organic components of the crustacean exoskeleton are chitin and protein, which are firmly associated with one another, Trim (1941), Stacy (1943) and Haworth (1946) regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of carbohydrate-containing amino sugars (chitin) with the protein. Further, since calcium is the most abundant, if not the most important, mineral constituent within the crustacean skeleton, an emphasis will be placed on the abundance and distribution of glycogen, phosphatase, and calcium in the integumental tissues. These three constituents, among others, are of extreme importance in the development and calcification of the new skeleton.

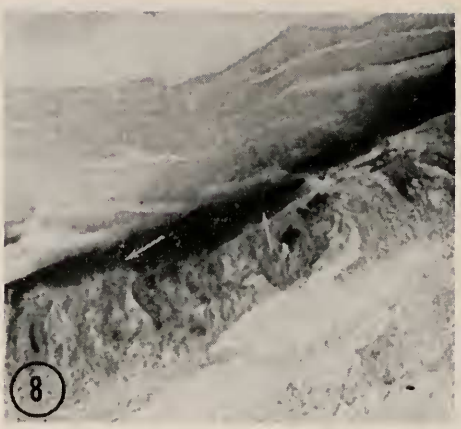
c. *Localization of glycogen, phosphatase and calcium*

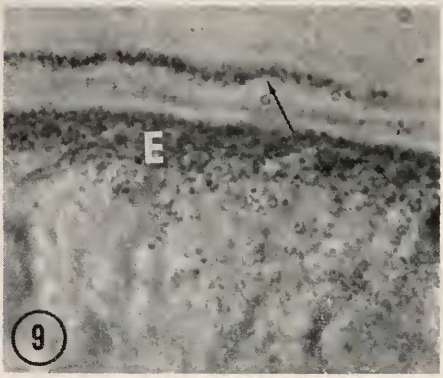
Glycogen: At *one day* following molt (Stage A), glycogen granules are scattered throughout the sub-epidermal connective tissue. Abundant amounts are concentrated at the bases of epidermal cells of the inner integument while the epidermal cells of the outer integument show little glycogen, which is localized in the proximal half of the cells (Fig. 4).

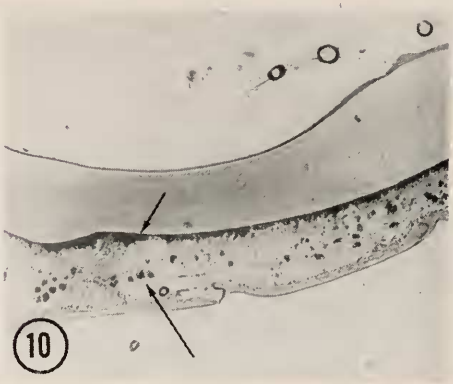
On the *second day* following molt, glycogen completely disappears from the inner epidermis (Fig. 5) and is not observed again in this tissue during the entire postmolt period. From two through four days following molt, little glycogen is apparent in the sub-epidermal connective tissues. Much heavier concentrations, on the other hand, are observed in the distal half of the outer epidermal cells (Fig. 6). By the *fifth day* glycogen accumulates in large amounts in the sub-epidermal tissues while the outer epidermis becomes almost depleted of it (Fig. 7). From the *sixth* through the *seventh day* most of the glycogen disappears from the sub-epidermal connective tissue and moves again to the outer epidermis where it is heavily concentrated in the distal portion of this tissue.

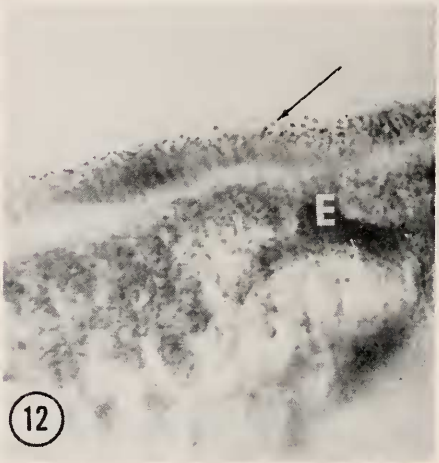
Phosphatase: Alkaline phosphatase becomes localized in the outer epidermis, being more heavily concentrated in the distal rather than the proximal half of the cells. Furthermore, the enzyme is heavily concentrated in the integument immediately above the epidermis (Fig. 8). It appears to be concentrated at this site in the region of the proximal portions of the innumerable pore canals. The localization of phosphatase in these sites is evident on the *first day following molt*, before calcification of the branchial integument begins, and remains in this localization throughout Stages A, B and very early C. In addition to its presence at these sites alkaline phosphatase is observed rather evenly distributed in the reserve cells during the entire period of observation. Control treated sections indicate that not only the enzyme but calcium as well are present in all of these sites from the second











FIGS. 7–12.

through the eighth day, although purpurin and alizarin red S did not show the presence of calcium within the reserve cells before the third day following molt. It might also be pointed out that the enzyme is likewise heavily concentrated around newly developing bristles.

Calcium: Calcification of the new outer integument of the branchiostegites begins on the second day following molt (Stage B). Before calcification begins, however, the epidermis begins to concentrate calcium in markedly evident amounts (detectable by purpurin, alizarin red S, Von Kossa's method, Gomori's (1941) method for alkaline phosphatase and confirmed by microincineration). Before discussing the calcification of the skeleton the author would like to point out that of these methods used for the detection of calcium, Von Kossa's method and Gomori's (1941) method for alkaline phosphatase are the most useful for showing actual stages in calcification of the integument. Furthermore, these two methods show calcium salts or complexes in granular form.

At *one day* following molt and one day before calcification of the integument begins, the distal ends of the epidermal cells begin to show calcium. No reserve cells of the integumental tissues, as will be recalled, bind calcium at this time.

By the *second day*, the distal portions of the epidermal cells are filled with calcium. The tissue spaces or sinuses at the base of the epidermis and the blood channels, sometimes observed between the epidermal cells, show the presence of calcium. Although only a few reserve cells are apparent, a few show scattered granular deposits of calcium. At this same period a narrow band of calcium granules appears in the new principal layer immediately distal to, but paralleling, the epidermis (Fig. 9). Likewise, immediately underlying the epicuticle and within the pigmented zone a heavy concentration of rows of granules is observed.

By the *third day* the epidermal cells as well as the reserve cells of the sub-epidermal connective tissue are completely filled with calcium (Figs. 10, 11) detected by all methods used for this purpose. The heaviest concentrations of calcium granules in the epidermis are observed in the distal half of the cell. Here they are observed to be extruded from the distal ends of the epidermal cells in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of cal-

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FIGURE 7. At five days following molt glycogen has disappeared from the outer epidermis but is heavily concentrated by the sub-epidermal connective tissue. 760 ×.

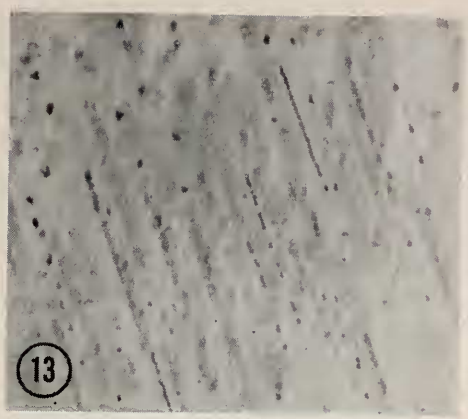
FIGURE 8. One day postmolt, before calcification begins in the branchial integument. Localization of alkaline phosphatase in the distal portion of the outer epidermis and in the integument immediately above the epidermis (region of the proximal portions of the innumerable pore canals, arrows). Phosphatase remains concentrated here through Stages A, B and early C. 420 ×.

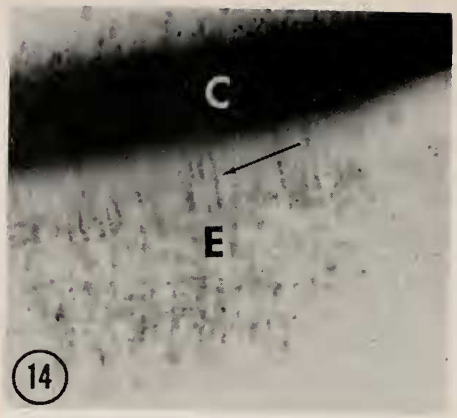
FIGURE 9. At two days postmolt calcification of the integument begins. Note that the distal portions of the outer epidermal cells (E) are filled with calcium and the narrow band of calcium granules (arrow) which appear in the newly forming principal layer immediately distal to, but paralleling, the epidermis. 2000 ×. Van Kossa's method.

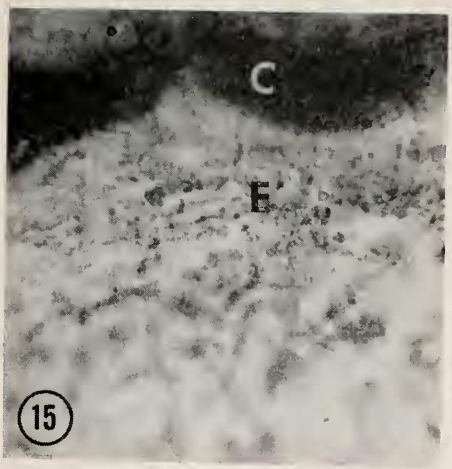
FIGURE 10. By the third day postmolt the epidermal cells (arrow) and the reserve cells (arrow) of the sub-epidermal connective tissue are filled with calcium. 90 ×.

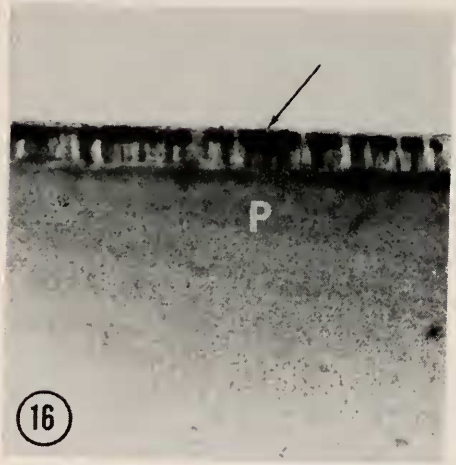
FIGURE 11. Higher magnification showing that the epidermal cells (E) and reserve cells (R) of sub-epidermal connective tissues, at three days postmolt, are filled with calcium. Note lumpy stainable balls of material in the reserve cells. 760 ×. Alizarin red S staining.

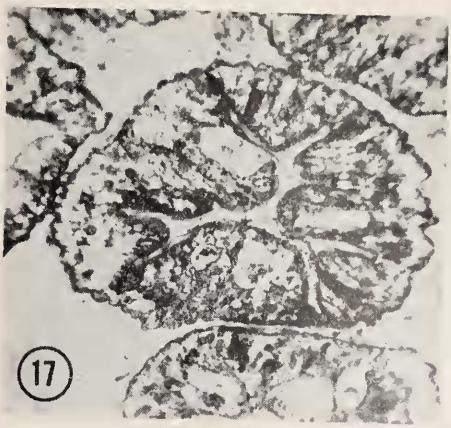
FIGURE 12. As the post-exuvial layers are deposited (three days following molt) masses of calcium granules are simultaneously extruded from the epidermal cells (E) to form narrow calcified bands paralleling the epidermis (arrow). 2000 ×. Van Kossa's method.

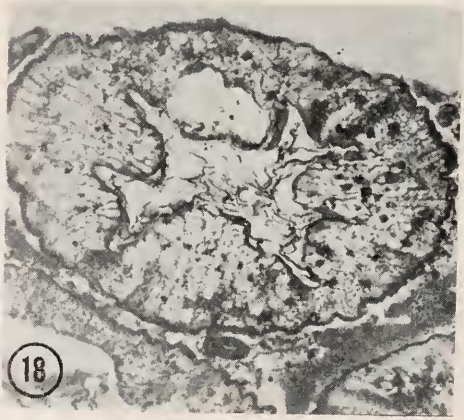












FIGS. 13–18.

cium granules are simultaneously extruded to form narrow bands paralleling the epidermis (Fig. 12). In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows. These rows of granules are particularly evident in the pigmented layer (Fig. 13) and can be seen to emanate from the epidermis (Fig. 14). The rows of granules correspond in location to the pore canals or vertical striae. Calcification of the pre-exuvial layers occurs after their formation. Hence, calcium has to be transported and deposited some distance from the epidermis. This transport, therefore, appears to occur through the pore canals.

From *four through seven days* calcium in the epidermis remains equal in amount to that observed on the third day.

Calcification continues to occur as the post-exuvial layers are deposited. As a consequence, the horizontal bands of granules paralleling the epidermis have thickened considerably by the end of Stage B (Fig. 15). By the *seventh day* portions of the inner integument are completely calcified. Calcification of the inner integument appears to occur in an identical fashion to that observed in the pre-exuvial layers of the outer integument.

2. *The hepatopancreas*

a. *Tissues*

The hepatopancreas, as discussed by Travis (1955a), is the major storage depot of organic and mineral reserves during Stage D and is, consequently, the major organ from which these reserves are mobilized when needed by other tissues during the postmolt period.

For five days following molt (Stage A and part of Stage B), the epithelial tissue of the hepatopancreatic tubules is predominantly of the absorbing type. This epithelial tissue consists of long tall columnar cells without large vacuoles and which may have either a central or basal nucleus (the so-called B_1 or R cells of Hirsch and Jacobs, 1928, 1930) as discussed by Travis (1955a). A few of the epithelial cells are of the secretory type (the vesicular or B_2 cells of Hirsch and Jacobs). These are enlarged swollen cells which enclose large vacuoles, some of

FIGURE 13. In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows corresponding in location to the pore canals. 2000 ×. Von Kossa's method.

FIGURE 14. Note narrow vertical rows of calcium granules, corresponding in location to the pore canals (arrow) emanating from the epidermis (E). Also note the rather wide band of calcium granules (C) in a newly formed portion of the principal zone paralleling the epidermis. 2000 ×. Von Kossa's method.

FIGURE 15. Horizontal bands of calcium granules paralleling the epidermis (E) continue to be deposited as the post-exuvial layers are deposited and a thick calcified band (C) in the new principal layer is noted by the end of Stage B. 2000 ×. Von Kossa's method.

FIGURE 16. By seven days following molt (end of Stage B) the epicuticle (arrow) and most of the pigmented layer (P) appear to be fully calcified except in certain areas where this is not complete. 2000 ×. Von Kossa's method.

FIGURE 17. Note the heavy concentration of glycogen at the basal and distal ends of the absorption cells and at the periphery of the vacuoles of secretory cells in the tubular epithelium of the hepatopancreas. One day following molt (Stage A). 200 ×.

FIGURE 18. At seven days following molt (end of Stage B) the tubular epithelium of the hepatopancreas is virtually devoid of glycogen. 200 ×.

which contain stainable material. These cells (Travis, 1955a) undergo apocrine breakdown in *Panulirus*. Their vacuolar contents plus adjacent cytoplasm are discharged into the lumen of the hepatopancreatic tubules, leaving only the basal region and nucleus of the cell intact. However, none of these observed secretory cells, during this period of five days following molt, show any sign of apocrine breakdown. This would be expected because the animals are still undergoing a period of inanition (Travis, 1954, 1955a, 1955b). Stainable material in the large vacuoles of the secretory cells frequently indicates the presence of lipid and mucopolysaccharide.

By the sixth and seventh day following molt, the predominant cell types observed in the epithelial tissue of the tubules are the secretory cells. On the seventh day there is much evidence of apocrine secretion within these cells. In general, most animals begin to feed again on the seventh day. This would not only account for the numerous secretory cells but also for the apocrine breakdown of many of these cells.

The large oval reserve cells are likewise apparent in the connective tissue between the individual tubules of the hepatopancreas. They similarly show, as do the reserve cells of the integumental tissues, the presence of phosphatase, mucopolysaccharide and calcium. Much lipid is also present during Stage A and B. From the fifth through the seventh day the reserve cells of the hepatopancreas show the presence of large vacuoles (Fig. 2), some of which contain flaky or granular-like stainable material while others appear to be clear. This is possibly correlated with a decrease in mucopolysaccharide, lipid, and calcium content in these cells. The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo cyclic peaks and declines in size and abundance, changes in structural appearance, and the storing and apparent release of reserves as indicated below.

During the *intermolt period* (late Stage C) they are numerous and large, ranging in size from 17–38 μ with an average size of 30 μ. They show markedly evident amounts of mucopolysaccharide. A similar situation prevails during the *premolt period*. The cells range in size from 17–38 μ with an average size of 32 μ and show large stainable spheres of material. At both of these stages much mucopolysaccharide is present. Calcium is likewise apparent in large amounts in the hepatopancreatic reserve cells, while the integumental tissue reserve cells are devoid of it during these periods.

By the *first day* following molt, there are few reserve cells present in the hepatopancreas and these have undergone a general decrease in size (8–29 μ, average size 16 μ), a situation opposite to that observed in the integumental tissues. Of the reserves, however, little calcium seems to be apparent and there is no evidence of spheres of material found within them. On the *second day* (beginning of Stage B) the cells remain few in number but have increased in size (range 24–38 μ, average size 31 μ), again a situation opposite to that observed in the integumental tissues, although no large spheres of material are evident. They again show the presence of small amounts of calcium. At *three days*, as in the integumental tissues, the reserve cells become numerous and remain large (range 24–48 μ, average size 30 μ). They take on a distinct "mulberry" appearance and show the presence of mucopolysaccharide and markedly evident amounts of calcium. A similar situation prevails on the *fourth day*. The cells in the hepatopancreas remain numerous and large (range 26–43 μ, average size 35 μ) and likewise show abundant mucopolysaccharide

and calcium. By the *fifth day* few cells are apparent and they are slightly smaller in size (21–48 μ, average size 28 μ). These cells become highly vacuolated, although some balls of material are present within the vacuoles, and show only a slightly pink coloration with PAS, a condition similar to that observed in the reserve cells of the integumental tissues. Little calcium is apparent. Except for the increase in number and slight increase in size (29–38 μ, average size 32 μ) the reserve cells on the *sixth day* remain similar to the *fifth day* condition though they contain large single vacuoles with no stainable balls of material. They remain numerous on the *seventh day* but the average size is slightly smaller (24–32 μ, average size 29 μ). Except for these changes they are similar to the *fifth day* condition with respect to reserves. It should be added that at all stages of the observation period, phosphatase and lipid are present.

Since different groups of animals were used for the study of hepatopancreatic and integumental tissues (fixed in different years), it is difficult to determine whether or not the cycles may be slightly out of phase at the sites of observation. The information at hand (Sewell, 1955; Travis, 1955a) strongly suggests that much more information is needed on these highly interesting and obviously important reserve cells.

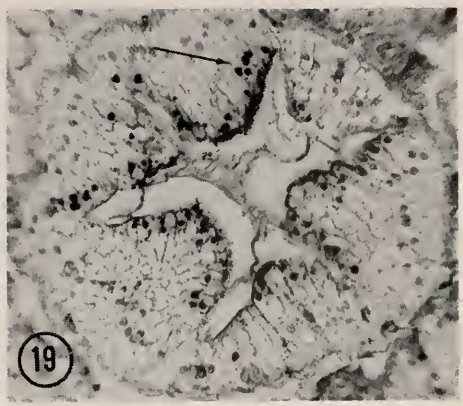
b. *Localization of glycogen, phosphatase, calcium and lipid*

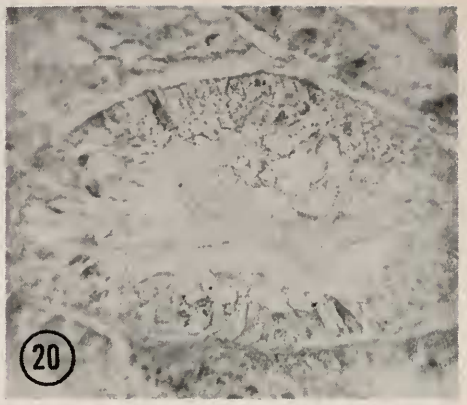
Glycogen: Although the concentration of glycogen within the tubular tissue on the first day following molt (Fig. 17) would compare with the premolt condition (see Figure 30; Travis, 1955a), there is a progressive decrease in abundance from the first through the seventh day. A decidedly marked decrease occurs by the sixth day and by the seventh day hardly a single granule of free glycogen can be detected within the tubular tissue (Fig. 18). During Stages A and B, glycogen is more heavily distributed in the distal and basal ends of the absorption cells. When mature secretory cells are observed during the early phase of Stage B, glycogen granules are localized at the periphery of the vacuoles and are sometimes observed within the lumen of the tubules.

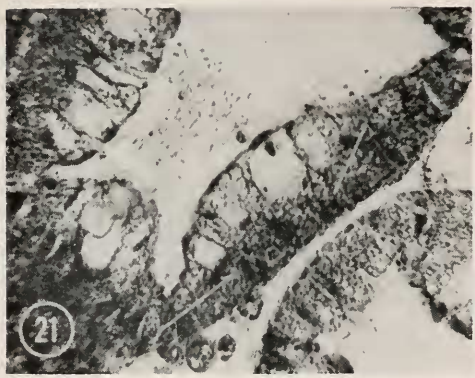
The disappearance of glycogen from the hepatopancreas by the seventh day might be expected because of the need of this constituent in the synthesis of the new skeleton. The integumental tissues, among others, therefore, accumulate and use large amounts of glycogen at the expense of the hepatopancreas. This is evident in both the premolt and postmolt period.

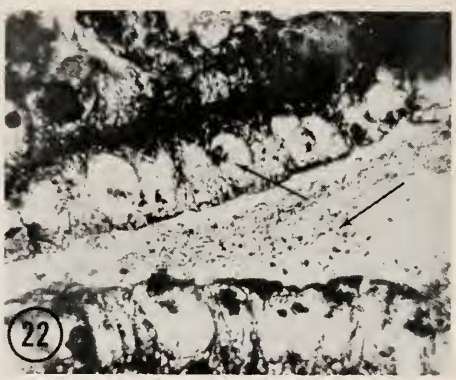
Phosphatase: During the early postmolt period alkaline phosphatase is almost absent from the striated borders of the tubular tissue but remains localized around the calcospherites (Fig. 19), which disappear progressively as calcification of the skeleton occurs. From the sixth through the seventh day when many large secretory cells are present, the enzyme is localized around the periphery of small and large secretory vacuoles. It is at all times present in the reserve cells.

Calcium phosphate: As in the premolt animals, the tubular epithelium of the postmolt animal is marked by the presence of innumerable calcospherites in the apical ends of the absorbing cells, very few being apparent in the small number of secretory cells. In the postmolt animal, however, these calcospherites disappear progressively as the skeleton is calcified. They have markedly decreased by the fifth day and by the seventh day (Fig. 20) hardly a single calcospherite can be de-









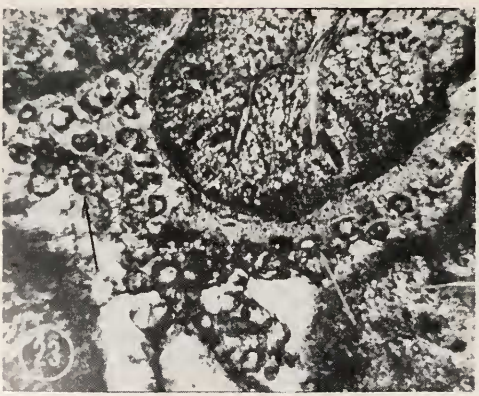


FIGURE 19. The tubular epithelium of the hepatopancreas at one day following molt (Stage A). Note alkaline phosphatase localized around innumerable calcospherites (arrow) at the apical ends of absorbing cells. 200 ×.

FIGURE 20. At seven days following molt, note that there is almost a complete absence of calcospherites and the enzyme alkaline phosphatase in the tubular epithelium of the hepatopancreas, a situation that occurs progressively as the integument is calcified. 160 ×.

tected. The complete disappearance of the calcospherites by the seventh day would be expected in *Panulirus* because these animals have to rely completely on this stored phosphate, stockpiled during the two-week premolt inanition period, as a source of phosphate for incorporation into the new skeleton. Since, as was pointed out previously (Travis, 1954, 1955a, 1955b), phosphate is obtained primarily from food, the stockpile of stored phosphate in the hepatopancreas would readily be depleted by the sixth or seventh day of starvation following molt. From low blood values following molt (Travis, 1955b) it is evident that the normal intermolt blood concentration within the body would not be replenished for at least three or four weeks following molt. Although the reserve cells of the hepatopancreas show the presence of calcium from one through seven days following molt, from the fifth through the seventh day little is apparent.

Lipid: For the entire observation period (Stages A and B) droplets of lipid are found throughout the epithelial tissue of the hepatopancreatic tubules. There would appear, by the sixth and seventh day, to be a decrease over that observed in the premolt animal, although much is still apparent. When secretory cells are apparent in great numbers (6 and 7 days) lipid droplets are frequently observed within the vacuoles (Figs. 21, 22). On the seventh day, when apocrine breakdown is evident, and when some of the animals begin to feed a little, lipid material becomes quite apparent within the lumen of the tubules, a condition which would be correlated with extracellular digestion of this constituent, as Van Weel (1955) has shown.

The reserve cells contain considerable quantities of lipid for the entire postmolt observation period (Fig. 23). There does appear to be a decrease over that observed during the intermolt (late Stage C) and the premolt period. Because of the presence of considerable quantities of lipid within the hepatopancreas at this time, a histochemical or a qualitative difference in amount is difficult, with certainty, to detect.

Discussion

The continued accretionary growth and hardening of the post-exuvial layers of the skeleton imposes upon the epidermis two major tasks, namely the synthesis and elaboration of the organic matrix and the simultaneous or accompanying elaboration of constituents for hardening the skeleton which may or may not alter the properties of the basic organic components, chitin and protein. The complexities of these two functions cannot be over-emphasized. Although the epidermis takes the lead in the performance of these tasks, the importance of other tissues, such as the hepatopancreatic and subepidermal tissues, cannot be under-estimated.

As the principal layer of the skeleton in *Panulirus* is deposited during the early postmolt period of observation (Stages A and B), the outer epidermis shows, tinctorially, that considerable amounts of a glyco- or mucoprotein are concentrated or

Figure 21. The distribution of lipid droplets within secretory cells of the hepatopancreas (arrows). The secretory cells become apparent in great numbers at six and seven days following molt. 200 ×.

Figure 22. Seven days following molt. Note lipid droplets (arrow) within the vacuoles of the secretory cells and within the lumen of the tubules (arrow). 200 ×.

Figure 23. Note that considerable quantities of lipid are bound by the reserve cells (arrows) of the hepatopancreas. 100 ×.

synthesized by this tissue. Similarly, during Stage A and most of B, the pigmented layer, one of the two pre-exuvial layers formed before molt, shows the presence of this same mucopolysaccharide. By the fifth day the pigmented layer no longer indicates a positive reaction for this constituent. There is a tinctorial change from gamma metachromasia (pink-purple) to beta metachromasia (deep purple) with toluidine blue, which indicates that the properties of the basic organic components, chitin and protein, have been altered. Possibly this is caused by considerable impregnation with calcium salts at this time or, more certainly, by quinones which form cross-linkages with the native protein phase of the cuticle (Pryor, 1940). The net result of this combination is the formation of a highly stable and insoluble product. As Krishnan (1951) pointed out, the tanning by quinones of the pigmented layer occurs in *Carcinus maenas* shortly following molt and this is followed by pigmentation at a slightly later period. It is, therefore, possible that both of these related processes are completed by the fifth day in *Panulirus*, thus causing this change in tinctorial properties.

The presence of muco- or glycoprotein in the epidermis for the entire postmolt observation period (Stages A and B) is doubtlessly related to the secretion and development of the principal layer, which is the only post-exuvial layer deposited during this period. This layer, like the pre-exuvial pigmented layer and post-exuvial membranous layer (formed and completed from the third through the fourth week following molt in *Panulirus*), consists as in insects of the basic organic components, chitin and protein, which are closely associated with one another. The firm combination of these two organic constituents has led Trim (1941), Stacy (1943) and Haworth (1946) to regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of the carbohydrate-containing amino sugars (chitin) with the protein. Richards (1951) has pointed out that the consideration of the arthropod cuticle as a double set of layers (the outer set being composed of lipoprotein and the inner set being composed of glyco- or mucoproteins) is advantageous. This consideration emphasizes that the major cuticular components seem to be formed and secreted as conjugated proteins and not as separate components.

The principal layer in *Panulirus* always shows, tinctorially, the presence of muco- or glycoproteins with the exception of the fact that immediately after each layer is deposited a green, rather than a pink, coloration with toluidine blue occurs. This suggests that the reactive groups are slightly altered following their immediate formation. Although the early stages in the formation of the membranous layer were not followed, the completed layer, in contrast to the principal layer, shows a green rather than a pink coloration, which indicates that this layer is, in some way, different from the principal layer. This is further revealed by the fact that calcium salts are never bound in this layer.

During the deposition of the principal layer of the outer integument glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis (Travis, 1951a, 1951c, 1955a) and in turn a shift from the hepatopancreatic tissues to these integumental tissues (see Observations). During the postmolt period of accumulation and utilization of glycogen by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears. Although it is abundant in the tubular tissue on the first day following molt, there is a marked decrease in glycogen by the

fifth day and hardly a single granule of free glycogen is present by the seventh day. The rhythmical accumulation by, and disappearance of, glycogen from the sub-epidermal tissues and similarly its accumulation by the epidermal cells and disappearance from the sub-epidermal tissues suggests a rhythmical cycle of accumulation and utilization in the epidermis, at the expense of sub-epidermal tissue glycogen. Likewise, the rhythmical accumulation of glycogen by the sub-epidermal tissue and progressive disappearance from the hepatopancreas similarly suggests that there are marked cycles of accumulation and utilization. These cycles of accumulation and utilization stem from the epidermis, which takes the lead in the elaboration of the post-exuvial layers of the integument, but also involves the hepatopancreas, which serves as the major storage organ from which such reserves can be mobilized and upon which the epidermis is ultimately dependent for the successful completion of its tasks. Since feeding begins on the seventh day following molt in the summer months, a constant supply of glycogen would be available to the epidermis until the integument is completed (late Stage C), but is not stockpiled in the hepatopancreas again until Stage D. The stockpiling of glycogen during this period of inanition would suggest strongly that the source of this constituent is from the large quantities of lipid reserves, likewise present in the hepatopancreas at this time. By the conversion of some lipid, through its glycerol moiety, to carbohydrate, the latter being stored as glycogen, the peak glycogen concentration could be achieved during Stage D. As was pointed out by Travis (1955a), evidence suggests (Renaud, 1949) that during periods of inanition (Stages D, A and B), lipids likewise serve as a major source of energy by playing a principal role in oxidative metabolism.

The periodic accumulation and utilization of glycogen by the epidermis as the post-exuvial layers are deposited suggests that glycogen is a necessary precursor for chitin formation. This possibility, as discussed by Travis (1955a), has been suggested by Verne (1924, 1926), Mataczyńska-Suchcitz (1948), Renaud (1949), Travis (1951a, 1955a) and Schwabe *et al.* (1952). Glycogen may likewise serve as a ready energy-source for the synthesis and elaboration of the organic constituents of the integument. This possibility has been suggested by Bradfield (1951). He found an abundance of glycogen in regenerating epidermis of the vertebrates. As the outermost cells keratinized, glycogen disappeared. He attributed this disappearance to the utilization of glycogen for the supply of energy in keratin synthesis. Glycogen may further serve indirectly as added substrate for phosphatase action after its hydrolysis and phosphorylation by phosphorylases. In this way, it has been postulated as one of the necessary mechanisms in calcification of bone and teeth of vertebrates (Robison and Soames, 1924; Harris, 1932; Glock, 1940; Horowitz, 1942; Engel, 1948; Marks and Shorr, 1950 and others). It is more likely, however, that glycogen participates in all of these functions and possibly others that have not been mentioned.

During the entire postmolt observation period (Stage A and D), alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells and is observed in the integument immediately distal to but paralleling the epidermis. The localization of the enzyme in this latter site is distinctly apparent by the first day following molt before any calcification begins in the branchial region of the integument. Krugler and Birkner (1948) noted a similar localization of the enzyme in the integument of the crayfish during premolt. In *Panulirus* this is a

strategic location for the enzyme during the postmolt period because it is in a region of high activity as the deposition and hardening of the post-exuvial layers occur. Further, it would appear that the enzyme may be specifically localized in the proximal portions of the pore canals. Because of its heavy concentration along this entire region of the integument, however, its specific localization in the pore canals is difficult to determine with certainty. The enzyme is likewise heavily concentrated in the integument around newly developing bristles. In the sub-epidermal tissues it is observed in the reserve cells.

In the hepatopancreas the most marked localization of the enzyme is seen around the innumerable calcospherites in the distal portions of the absorption cells. The enzyme is observed in the striated borders of these cells and on the sixth and seventh day, when a predominance of secretory cells is evident, it is observed at the periphery of small and large vacuoles. The reserve cells within the blood or tissue spaces between the tubules of the hepatopancreas likewise show the presence of the enzyme.

As in the premolt animal, phosphatase is localized around the calcospherites in the absorption cells of the hepatopancreas and since these disappear progressively as calcification of the post-exuvial layers occurs, it is possible that the enzyme participates in the mobilization of this reserve for transfer to the integument. It may do so by dephosphorylating, in some way, the precipitated complex. In this role, it would be serving in resorption at this site and could at the same time be involved in mediating the synthesis of other phosphoric esters to be conveyed via the blood from the hepatopancreas to the integumental tissues. That this indeed may be an important function of phosphatase in bone resorption has been suggested by McLean and Urist (1955). Further, its localization around the periphery of small and large vacuoles on the sixth and seventh day would suggest that the enzyme is possibly involved, in some way, with the synthesis of secretory products or the transfer of these products from the adjacent cytoplasm into the secretory vacuoles. Phosphatase localization at the striated borders of the absorption, as well as the secretory, cells would suggest that when these cells are active the enzyme would likewise serve the function of participating in transfer reactions by producing molecules which enter or leave the cells more readily. Such a function has been suggested by Moog (1946).

The concentration of the enzyme at the distal ends of the outer epidermal cells and in the integument immediately distal to, but paralleling, the epidermis suggests its extremely important functions in the deposition and hardening of the post-exuvial layers. The periodic accumulation and utilization of glycogen by the epidermis, as was pointed out earlier, would suggest that possibly this constituent, glycogen, is used as a precursor in chitin formation. If this is so, and if the synthesis of chitin occurs, as Renaud (1949) suggested, by the hydrolysis of glycogen and dephosphorylation of glucose phosphate to glucose, this step being followed by subsequent steps to yield chitin, phosphatase would play an important role in this chain of events by its dephosphorylation of glucose phosphate to glucose, a possible starting point for chitin formation. Likewise, if glycogen were used as an energy-source for the synthesis and elaboration of the organic matrix, phosphatase would be intimately involved in these reactions. Glycogen, as suggested earlier, could serve indirectly as added substrate for phosphatase action. The distribution of phosphatase and mucopolysaccharide in the epidermis and its distribution in the

region of the newly forming post-exuvial layers of the integument, immediately above the epidermis, suggests that it may play a very important part in the formation of the ground substance (mucopolysaccharide) of the post-exuvial layers. Furthermore, the enzyme is thought to play an important role in the manufacture of fibrous proteins, thus participating in the formation of the ground substance of bone (McLean and Urist, 1955). Moog and Wenger (1952), however, have suggested that since the enzyme and mucopolysaccharide are frequently found together in fibrous structures, the mucopolysaccharide constitutes part of a cytoskeletal mechanism to which the enzyme is bound.

The appearance of alkaline phosphatase in the integument immediately distal to and paralleling the epidermis one day before calcification begins, likewise suggests to the author that the enzyme is intimately involved in calcification of the integument. In such a localization it could provide a mechanism for the production of a local high concentration of phosphate ions. In the presence of calcium ions, transferred across the cell membranes of the epidermis, phosphate could then unite to form the calcium salt, calcium phosphate, which constitutes about 3% of the total

TABLE I

Analyses to indicate the amount of mineral and organic matter in the entire area of softening of a late Stage C animal

Substance analyzed	Per cent present in the area of softening	Calculated % of salts present	
CaO	24.64	$CaCO_3$	42.39
MgO	1.98	$MgCO_3$	4.04
P_2O_5	1.40	$Ca_3(PO_4)_2$	3.05
CO_2	21.39	Carbonates unaccounted for	2.22
Per cent mineral as Ca, P, and Mg oxides	28.02		
Per cent organic matter	71.98		

mineral salt of the integument (Table I). The almost certain presence of phosphorylases at these sites, although not specifically determined, would likewise be expected to be important in the calcification of the integument, by synthesizing potential substrates for phosphatase action in zones of calcification.

Hardening of the crustacean skeleton occurs by quinone tanning and calcification. Hardening by quinones is a result of the oxidation of polyphenols to quinones, which form cross linkages with the native protein of the cuticle. The net result of this combination is a highly stable and highly insoluble product. Of the pre-exuvial layers, the epicuticle of *Carcinus maenas* is hardened by quinones shortly after its formation, whereas subsequent hardening of the pigmented layer occurs soon after molt (Krishnan, 1951). As was suggested in a previous section of the discussion, changes in tinctorial properties of the pigmented layer suggest that the process of hardening by quinones is complete by the fifth day following molt. Quinone tanning, although the primary cause of hardening in the exoskeleton of insects, plays a much smaller role in Crustacea (Dennell, 1947), calcification being the major cause of hardening.

Calcification begins on the second day following molt in *Panulirus* and occurs thereafter simultaneously with or immediately accompanying the elaboration of layers of the principal zone. Further, the additional task posed to the epidermis is that of calcifying the *pre-exuvial layers;* calcification, in this case, is of course a process that is accomplished long after their formation but during the same time at which the post-exuvial layers are being calcified.

In the distal region of the epidermal cells, where calcium becomes most heavily concentrated, extrusion of calcium from this tissue occurs in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows. These rows, as pointed out earlier, are particularly evident in the pigmented layer, and are likewise observed to emanate from the epidermis. They correspond in location to the pore canals or vertical striae, protoplasmic extensions of the epidermis. Thus, in the case of the pre-exuvial layers, calcification occurs after their formation. Hence, calcium must be transported and deposited some distance from the newly forming pre-exuvial layers. This transport occurs through the pore canals, thus enabling the epidermis to act at these distant sites.

Calcification of the integument continues and is almost entirely completed by the seventh day (end of Stage B) in the epicuticle and most of the pigmented layer.

While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storage, possibly alternating with cyclic release to the epidermis. If calcium in these cells is used periodically by the epidermis, which it probably is, the reserve cells could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. At no time during Stage A and B, however, are the epidermal cells depleted of calcium. This might be expected because the concentration of calcium in the blood (Travis, 1951b, 1955b) is sufficiently high to provide a continued supply of this element to the epidermis. Previous reference to the reserve cells has already been made as to their cyclic peaks and declines in abundance and size, change in structural appearance and in the binding and release of reserves, other than calcium (see Observations) and will not therefore be discussed in this section.

As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. Though abundant on the first day following molt they progressively decrease in number as calcification of the new integument occurs and by the seventh day hardly a single calcospherite is to be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton (Travis, 1955a), represent a major source, therefore, from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed. Since the spiny lobster obtains most of its phosphorus from food and since the animals do not feed for two weeks before molt, when resorbed phosphorus from the old skeleton is being stockpiled in the hepatopancreas (Travis, 1955a), one would expect a depletion of this mineral reserve during the first week following molt as calcification of the new integument occurs. This depletion is also evidenced by the low blood-phosphorus levels following molt (Travis, 1955b).

It is interesting that the reserve cells of the hepatopancreas show the presence

of calcium from one through seven days following molt but from the fifth through the seventh day little is apparent. The fifth through the seventh day is a period when these cells become vacuolated, which may be correlated with the apparent decrease in mucopolysaccharide, lipid and calcium. It is possible that as the calcium phosphate is mobilized from the calcospherites it is immediately transferred from the absorption cells to the reserve cells and from these to carriers in the blood, possibly organic acids of oxidative and glycolytic metabolism. The evidence at hand, however, is not sufficient at this time to determine whether there is an actual movement of the reserve cells from the hepatopancreatic tissues to the integumental tissues.

Little has been said about the development and hardening of the inner integument during Stages A and B. It will be recalled that glycogen is observed in abundance at the bases of the epidermal cells of the inner integument on the first day following molt (Stage A) but completely disappears from the inner epidermis by the second day, not to be observed again at this site for the entire observation period. Similarly, no further thickening of this integument occurs after the second day following molt, indicating that the development of the inner integument of the branchiostegites is completed during a period of three days preceding molt and two days following molt (Travis, 1955a). This does not mean, however, that hardening by calcification is completed at this time. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation. By the fourth day the uniformly staining endocuticle of the inner integument has begun to calcify and by the seventh day portions of it have completely calcified. Calcification in the inner integument occurs in the same fashion as that observed in the pre-exuvial layers of the outer integument, *i.e.*, via the port canals.

The two major tasks which must be achieved by the epidermis, namely, the synthesis and elaboration of an organic matrix and the simultaneous or accompanying elaboration of calcium salts for hardening of the newly developing skeleton, are not completely accomplished before the fourth week following molt during the summer months. Calcification is probably completed by the third week following molt, a time at which the membranous or non-calcified layer begins to be formed. This layer is not fully completed before the fourth week following molt (late Stage C or C_4 of Drach, 1939). Approximately three weeks following the completion of the integument the epidermis is again confronted with the preparation for growth in size of the animal (Travis, 1955a).

The cells of the epidermis, like the osteoblasts of bone, synthesize and elaborate the organic matrix of the skeleton and, unlike the osteoblasts of bone, they actually concentrate and secrete the mineral constituents, principally calcium, which are precipitated in the matrix. Furthermore, the epidermis, like the osteoclasts of bone, participates intimately in processes of resorption of the integument. It elaborates the proteinases and chitinases which break down the organic matrix. It likewise resorbs these organic breakdown products along with the mineral constituents and also participates in their transfer across its cell membranes to the blood for further handling (Travis, 1955b).

Following molt the transfer of calcium ions from the blood across the cell membranes of the epidermis and the concentration of calcium by this tissue is truly remarkable. Within the epidermal cells, the calcium is doubtlessly immobilized ionically by the binding capacity of weakly acidic groups of protein, succinate, lactate, bicarbonate, phosphate, citrate or by other anionic groups. On release of calcium

to the exterior of the cell, an alteration in the binding capacity of anionic groups is necessary. After release from the epidermis, calcium is precipitated as salts, by various mechanisms, in the organic matrix of the integument.

The distribution of alkaline phosphatase in the distal portion of the epidermis and particularly in the integument immediately distal to and paralleling the epidermis suggests that this enzyme would provide a mechanism for the local high concentration of phosphate ions, which in the presence of some of the calcium released from the epidermis could account for the precipitation of the 3% calcium phosphate of the integument (Table I).

Calcium carbonate, however, is the principal salt of the spiny lobster skeleton, and Crustacea in general, and constitutes approximately 42% of the total mineral deposited in the skeleton of *Panulirus*. It is, therefore, of interest to point out a related and possibly important enzyme involved in calcification of the skeleton. This is the enzyme, carbonic anhydrase. Sabotka and Kann (1941) found that this enzyme was not present in the gills of *Panulirus argus*. Because of this, they suggested that elimination of CO_2 is not confined to the gills, but that the bicarbonate formed may be eliminated in the skeleton by precipitation as $CaCO_3$. Maluf (1940) found that considerable quantities of carbonic anhydrase were present in the epidermis and skeleton of the crayfish, *Cambarus clarkii* and the American lobster, *Homarus americanus*. The fact that alkaline phosphatase is found in the distal ends of the epidermal cells and integument immediately above and paralleling the epidermis would suggest that the hydrogen ion concentration is low and the pH is on the alkaline side (between 8–10). At this pH, dissociation of bicarbonate into $CO_3^=$ and H^+ would be expected. In the presence of calcium ions released from the epidermis, the precipitation of calcium carbonate could occur. Thus, again the mechanism and conditions exist for the production of local high concentrations of bicarbonate and carbonate ions in the epidermis and integument of Crustacea.

The manner in which calcium carbonate is precipitated, *i.e.*, as calcite rather than aragonite or amorphous calcium carbonate, is undoubtedly determined by conditions inherent in the organic matrix which favor calcite precipitation. Prenant (1927), however, pointed out that the condition determining the state of calcium carbonate precipitation is the proportion of phosphates to carbonates, as indicated by the P_2O_5/CO_2 ratio. If the ratio is more than 0.015, calcium carbonate is deposited in amorphous form. If the ratio is 0.105 or less, the calcium is deposited in crystalline form. The P_2O_5/CO_2 ratio in *Panulirus* is 0.0657. Calcium carbonate in *Panulirus* is precipitated as calcite, which is consistent with the idea proposed by Prenant. This would not mean, however, that conditions in the organic matrix do not favor calcite, rather than aragonite, precipitation.

That the calcification mechanism in *Panulirus* may be influenced by the presence of organic acids such as lactic, succinic, and citric acid at the sites of calcification has yet to be investigated. These acids have a marked propensity for forming weakly ionized salts with calcium. It is highly likely, therefore, that, as in the calcification of bone, citric acid plays an important role in the calcification mechanism of Crustacea. It is known from the work of Dickens (1941) that more than 70% of the citric acid of the human body is in the skeleton and that as much as 1% of the fresh weight of bone may be accounted for as citrate. It is not definitely known whether citrate is present as an ion or precipitated as a calcium citrate complex. Bones examined for enzymes of the citric acid cycle have shown that by comparison

with other tissues, such as the kidney or liver, citrogenase and aconitase activities are much greater than those of isocitric dehydrogenase (McLean and Urist, 1955). As these authors point out, the mechanism for the production of local high concentrations of citric acid exists in bone. Other than the work of Thunberg (1949), however, as quoted from Thunberg (1953) and Steinhardt (1946), nothing has been done with the role of citric acid in the calcification process of the Crustacea. Thunberg found that at least 0.8% of the gastroliths of a European crayfish was constituted of citric acid. This would suggest strongly that citric acid is involved, as in bone, in some way in the calcification process. Steinhardt (1946) has further pointed out that in structures such as bone and gastroliths, where high concentrations of citric acid are found, the phosphorus and calcium content is also high. In such cases, citrate probably exists in a complex form in which calcium, phosphoric and citric acid enter.

It is highly likely, therefore, that within the integument of Crustacea, as in the gastroliths of the crayfish, the mechanism for the production of local high concentrations of citric acid exists. If so, the citrate produced, having a marked propensity for forming weakly ionized salts with certain cations such as calcium, could combine with calcium released from the epidermis and could enter into the mineral complex of calcium salts of the integument. If this is the case, increases and decreases of citrate formed enzymatically within tissues may be one of the regulators of ionic calcium activity. Furthermore, in normal metabolism, the normal activity of tissues may be controlled, in part, by the interaction of ionic calcium with citrate reached via the tricarboxylic cycle. Such a possibility has been suggested for the mammal by Peters (1950).

It is apparent from the foregoing discussion that the continued growth of the skeleton in an accretionary manner and the hardening of it by calcification, two of the major tasks confronting the crustacean from Stage A to late Stage C, are indeed complex. Although these extraordinary duties are put to the epidermis, which takes the lead in the performance of them, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

Summary

1. During the early postmolt period (Stages A and B) as rapid accretionary growth and calcification of the skeleton are occurring, changes are observed in the hepatopancreas and integumental tissues.

2. As the principal layer of the skeleton in *Panulirus* is deposited during Stage A and B, the outer epidermis concentrates or synthesizes a considerable amount of glyco- or mucoprotein, which is probably related to and involved in the secretion and development of this layer. Similarly, the pigmented layer, one of the preexuvial layers formed before molt, shows the presence of this same mucopolysaccharide. Near the end of Stage B, however, the properties of the basic organic components in the pigmented layer have been altered, possibly by considerable impregnation with calcium salts or by quinones.

3. During the deposition of the principal layer of the outer integument, glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis and in turn a shift from the hepatopancreatic tissues to the integumental tissues. During this period

of accumulation and utilization by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears and by the end of Stage B none remains.

4. The possibility of lipid conversion to carbohydrate, and the storing of this as glycogen in the hepatopancreas during Stage D, is discussed. The utilization of glycogen by the epidermis during Stages A and B, periods of inanition, is also discussed.

5. During Stages A and B alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells. It is observed in the integument in the region of the proximal portions of the pore canals, even before calcification begins. This is a region of high activity as deposition and hardening of the post-exuvial layers occurs. The enzyme is likewise found in the reserve cells of the subepidermal cells. In the hepatopancreas, the most marked localization of the enzyme is seen around the innumerable calcospherites in the absorption cells and in the striated border of these cells. The reserve cells of the hepatopancreas likewise show the presence of the enzyme. Functions of phosphatase in these sites are suggested.

6. Calcification begins the second day following molt and occurs thereafter simultaneously with, or immediately accompanying, the elaboration of layers of the principal zone. Calcification of the pre-exuvial layers, formed before molt, is a process accomplished long after their formation but during the same period at which the post-exuvial layers, formed after molt, are being calcified.

Calcium, heavily concentrated in the distal region of the epidermal cells, is extruded from this tissue in two distinct ways. As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows which emanate from the epidermis. These vertical rows of calcium granules correspond in location to the pore canals. Since calcification of the pre-exuvial layers occurs after their formation calcium must be transported and deposited some distance from the newly forming post-exuvial layers. This transport occurs through the pore canals, protoplasmic extensions of the epidermis, thus enabling this tissue to act at these distant sites.

7. While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storing alternating with cyclic release to the epidermis. The reserve cells in this capacity could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. Furthermore, these interesting reserve cells, during the early postmolt period (Stages A and B), undergo at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties and the storing of reserves other than calcium. The mucopolysaccharide material, either muco- or glycoprotein, in the reserve cells disappears near the latter part of Stage B. This indicates a decrease in the concentration of the material and suggests that the mucopolysaccharide stored by the reserve cells represents reserve material for the construction of the new skeleton.

The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo during the early postmolt period (Stages A and B) cyclic peaks and declines in number, size and the storing and apparent release of reserves. They similarly show the presence of phosphatase, mucopolysaccharide, calcium and much lipid.

8. As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. These calcospherites, abundant preceding molt and on the first day following molt, progressively decrease in number as calcification of the new integument occurs and by the seventh day (end of Stage B) hardly a single calcospherite can be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton, probably represent a major source from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed.

9. Development of the inner integument of the branchiostegites is completed in *Panulirus* during a period of three days preceding molt and two days following molt. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation via the pore canals, and portions of this integument are completely calcified by the seventh day following molt (end of Stage B).

10. Calcium carbonate, the principal salt of the spiny lobster skeleton, constitutes approximately 42% of the total mineral deposited and is precipitated in the organic matrix as calcite, rather than aragonite or amorphous calcium carbonate.

11. The roles of carbonic anhydrase and citric acid in the calcification of the integument of Crustacea are discussed.

12. Continued accretionary growth of the skeleton and the hardening of it by calcification are two major tasks confronting the crustacean from Stage A to late Stage C. Although the epidermis takes the lead in the performance of these duties, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

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